UNFOLDING THE ROLE OF PROTEIN MISFOLDING IN NEURODEGENERATIVE DISEASES

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Recent evidence indicates that diverse neurodegenerative diseases might have a common cause and pathological mechanism — the misfolding, aggregation and accumulation of proteins in the brain, resulting in neuronal apoptosis. Studies from different disciplines strongly support this hypothesis and indicate that a common therapy for these devastating disorders might be possible. The aim of this article is to review the literature on the molecular mechanism of protein misfolding and aggregation, its role in neurodegeneration and the potential targets for therapeutic intervention in neurodegenerative diseases. Many questions still need to be answered and future research in this field will result in exciting new discoveries that might impact other areas of biology.

CHAPERONE PROTEINS
A family of cellular proteins that mediate the correct folding of other polypeptides, and in some cases their assembly into oligomeric structures, but which are not components of those final structures. It is believed that chaperone proteins assist polypeptides in folding by inhibiting alternative assembly pathways that produce nonfunctional structures.

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To ensure that cells and organisms function properly, the correct activity of a network of thousands of proteins is essential. The function of a protein depends on its three-dimensional structure, which is determined by its amino-acid sequence. CHAPERONE PROTEINS supervise PROTEIN FOLDING so that, in most cases, mistakes are avoided and malfunctioning proteins are removed. However, evidence is accumulating that protein misfolding and aggregation is the most likely cause of various neurological and systemic diseases. These PROTEIN CONFORMATIONAL DISORDERS[1–3] include the most common forms of neurodegenerative disease as well as some rare inherited disorders that involve deposition of protein aggregates in the brain.

Neurodegenerative diseases can affect abstract thinking, skilled movements, emotional feelings, cognition, memory and other abilities[4]. This diverse group of diseases includes Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) (and related polyglutamine disorders including several forms of spinocerebellar ataxia or SCA), transmissible spongiform encephalopathies (TSEs, which include several human and animal diseases) and amyotrophic lateral sclerosis (ALS) (TABLE 1). Until recently, it was considered impossible to find a common molecular mechanism among this group of diseases. However, despite their obvious differences in clinical symptoms and disease progression, these disorders do share some common features: most of them (except HD and SCA) have both sporadic and inherited origins, all of them appear later in life (usually after the fourth or fifth decade), and their pathology is characterized by neuronal loss and synaptic abnormalities[4].

The hallmark feature of conformational disorders is that a particular protein can fold into a stable alternative conformation, which in most cases results in its aggregation and accumulation in tissues as fibrillar deposits[1–3]. These deposits have some similar morphological, structural and staining characteristics, but it is likely that different protein deposits might also have distinct biochemical or biological features, particularly depending on whether the aggregates accumulate intra- or extracellularly. The name AMYLOID was originally used to refer to the extracellular protein deposits found in AD and systemic amyloid disorders, but its use has recently been extended to include some intracellular aggregates. In this article I use the term amyloid-like deposits to refer to these aggregates, without meaning that they are absolutely equivalent. Amyloid is a generic term that refers to aggregates organized in a cross-β structure, with specific tinctorial properties (binding to Congo red and thioflavin S), higher resistance to proteolytic degradation

Table 1 | Clinical, pathological and biochemical features of neurodegenerative diseases

Disease	Mode of transmission	Clinical features	Affected regions of the brain	Protein involved	Cellular location of aggregates
Alzheimer's	Sporadic (95%) or inherited (5%)	Progressive dementia	Hippocampus, cerebral cotex	Amyloid-β and tau	Extracellular, cytoplasmic
Parkinson's	Mostly sporadic, rarely inherited	Movement disorder	Substantia nigra, hypothalamus	α-Synuclein	Cytoplasmic
Huntington's	Inherited (autosomal dominant)	Dementia, motor and psychiatric problems	Striatum, cerebral cortex	Huntingtin	Nuclear
Amyotrophic lateral sclerosis	Sporadic (90%) or inherited (10%)	Movement disorder	Motor cortex, brainstem	Superoxide dismutase	Cytoplasmic
Transmissible spongiform encephalopathies	Sporadic (90%), inherited (8%) or infectious (2%)	Dementia, ataxia, psychiatric problems or insomnia	Various regions depending on the disease	Prion protein	Extracellular

and a fibrillar appearance under electron microscopy (straight, unbranched, 10 nm wide fibrils).

In this article, I review recent data indicating that a potential mechanism of neurodegeneration, involving protein misfolding and aggregation, is a common cause of neurodegenerative diseases. I focus mainly on five neurodegenerative diseases (AD, PD, HD, TSE and ALS), but findings from other protein conformational disorders will also be used to illustrate some sections.

Cause, consequence or epiphenomenon?

The first indication that protein misfolding and aggregation were involved in neurodegenerative diseases came from post-mortem neuropathological studies (FIG. 1). Almost a century ago, Alois Alzheimer described the typical neuropathological hallmarks of the disease that takes his name: neuritic amyloid plaques and neurofibrillary tangles. Amyloid plaques are deposited extracellularly in the brain parenchyma and around the cerebral vessel walls, and their main component is a 40- or 42-residue peptide — amyloid-β protein (Aβ)[5]. Tangles are located in the cytoplasm of degenerating neurons and comprise aggregates of hyperphosphorylated tau protein[6]. In patients with PD, the cytoplasm of neurons from the substantia nigra contains aggregates called Lewy bodies[7]. The major constituents of these aggregates are fragments of a protein named α-synuclein[8]. Intranuclear deposits of a polyglutamine-rich version of huntingtin protein are a typical feature of brains from patients with HD[9]. In ALS, patients have aggregates, mainly composed of superoxide dismutase (SOD1), in the cell bodies and axons of motor neurons[10]. Finally, the brains of humans and animals that have been affected by various forms of TSE are characterized by the accumulation of protease-resistant aggregates of the prion protein (PrP), sometimes in the form of deposits similar to the amyloid plaques of AD[11].

Although cerebral aggregates have been recognized as a typical feature of neurodegenerative diseases for many years, neuropathological studies have been unable to determine whether they are directly involved in the pathogenesis of the diseases. Post-mortem studies that show a poor correlation between the load of amyloid-like deposits in the brain and the severity of clinical symptoms[12,13] are often used to argue against a primary role of protein aggregates in neurodegeneration. Moreover, the mixture of different types of aggregates in some diseases[14,15] and the appearance of protein deposits in clinically normal people[7,16] further confuse the neuropathological link between protein aggregation and disease. One possible interpretation of these findings is that protein aggregates have different roles in distinct diseases.

Support for a causal role of protein misfolding in neurodegenerative diseases has come more recently from genetic studies[15]. Mutations in the genes that encode the protein components of fibrillar aggregates are genetically associated with the inherited forms of all neurodegenerative diseases. The familial forms usually have an earlier onset and greater severity than sporadic cases and they are also associated with a greater load of protein aggregates[15]. Mutations in the respective fibrillar proteins have been found in AD, TSE, HD and related polyglutamine disorders, PD and ALS[17–21].

The generation of transgenic animal models bearing mutant forms of the human genes encoding the fibrillar protein have provided good evidence for the contribution of protein misfolding to disease pathogenesis. Several pathological and clinical features of diverse neurodegenerative diseases have been observed in transgenic models in which protein aggregates were successfully produced. Transgenic mice that overexpress high levels of mutated human amyloid precursor protein (APP), from which Aβ is generated, progressively develop many of the pathological hallmarks of AD, including cerebral amyloid deposits, neuritic dystrophy, ASTROGLIOSIS, and cognitive and behavioural alterations[22]. Transgenic mice expressing the wild-type human gene for α-synuclein develop several of the clinicopathological features of PD, including accumulation of Lewy bodies in neurons of the neocortex, hippocampus and substantia nigra, the loss of dopaminergic terminals in the basal ganglia and associated motor impairments[23]. ALS-like pathology occurs in mice bearing the human mutated SOD1 gene[24]. These mice develop motor neuron dysfunction and typical pathological alterations, including the presence of hyaline inclusion bodies in degenerating axons, muscle atrophy, astrocytic damage and the extensive loss of

PROTEIN FOLDING
The process by which a protein acquires its native tridimensional structure. Under physiological conditions, each protein has a unique stable folded structure, but in conformational disorders the polypeptide chain adopts an alternative structure, associated with the pathogenesis of the disease.

PROTEIN CONFORMATIONAL DISORDERS
A recently recognized group of diseases in which the key event is the misfolding, aggregation and tissue deposition of a protein.

AMYLOID
A general term for a variety of protein aggregates that accumulate as extracellular fibrils of 7–10 nm and have common structural features, including a β-pleated sheet conformation and the ability to bind such dyes as Congo red and thioflavins S and T.

ASTROGLIOSIS
Proliferation and ramification of glial cells in response to brain damage.

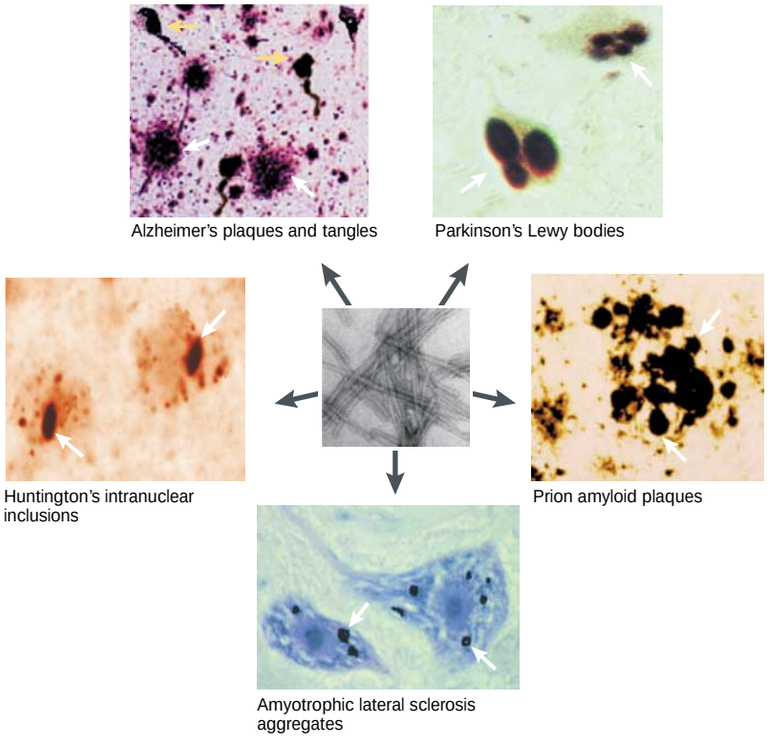

Figure 1 | **Cerebral aggregates in neurodegenerative diseases.** Extracellular amyloid plaques (white arrows) and intracytoplasmic neurofibrillary tangles (yellow arrows) are the pathological signature of Alzheimer's disease. Intracytoplasmic aggregates are typically present in the neurons of people affected by Parkinson's disease and amyotrophic lateral sclerosis. Intranuclear inclusions of huntingtin are observed in Huntington's disease patients and extracellular prion amyloid plaques that are located in different brain regions are present in some cases of transmissible spongiform encephalopathy. In spite of the different protein compositions, the ultrastructure of these deposits seems to be similar and composed mainly of a network of fibrillar polymers (centre).

large myelinated axons of motor neurons[24]. Transgenic mice containing exon 1 of human huntingtin, with 115–156 CAG repeat expansions, develop pronounced neuronal intranuclear inclusions, containing huntingtin and ubiquitin, before developing progressive neurological dysfunction with a movement disorder and weight loss similar to that associated with HD[25,26]. Overexpression of the human mutated *PrP* gene in mice results in spontaneous neurological disease with SPONGIFORM DEGENERATION of the brain similar to that seen in natural cases of TSE[27]. The fact that some clinicopathological features of diverse neurodegenerative diseases have been produced by the sole insertion into the mouse genome of the human gene for the protein that undergoes misfolding and aggregation supports the importance of this process. However, in some animal models of AD, TSE and ataxias, cerebral damage and clinical symptoms have been observed in the absence of detectable protein aggregates[28,29]. Moreover, some forms of TSE in animals and humans are not associated with detectable aggregates. These findings suggest that misfolded oligomeric forms or small aggregates that are not deposited in the tissue might be the culprits of neurodegeneration.

SPONGIFORM DEGENERATION
The brain damage associated with TSE or prion diseases, consisting of extensive vacuolization of neuronal cells.

β-SHEETS
β-Sheets and α-helices are the two types of prevalent, repetitive secondary structure in folded proteins. β-Sheets are formed of alternating peptide pleated strands linked by hydrogen bonding between the NH and CO groups of the peptide bond. Formation of β-sheets can be stabilized by protein oligomerization or aggregation.

Strong support for a key role of protein misfolding in disease comes from studies of the transmissibility of TSEs and the nature of the infectious agent. TSEs, also known as prion disorders, include Creutzfeldt–Jakob disease, fatal familial insomnia, Gerstmann–Straussler syndrome, bovine spongiform encephalopathy and scrapie. A unique characteristic of TSEs is that the same disease can have sporadic, inherited and infectious origins. The TSE infectious agent, known as the prion, consists mainly (or exclusively) of misfolded PrP, which is called PrP^{Sc} to differentiate it from the normally folded protein, PrP^{C} (REF. 30). The fact that the full pathological and clinical characteristics of prion diseases can be propagated from individual to individual by serial passage of pure PrP^{Sc} (REF. 30) is a good indication that the misfolding of the prion protein is the most likely cause of the disease. Transmission depends on the conversion of host PrP^{C} into the misfolded protein, which is induced by infectious PrP^{Sc} (REF. 30). None of the other protein conformational disorders has been convincingly shown to be transmissible. However, in animal models of AD and systemic conformational disorders that are associated with the deposition of amyloid-A in spleen and apolipoprotein A-II in several organs, the disease pathology can be accelerated by the injection of tissue homogenate enriched in the misfolded protein[31–33]. Other examples of propagation of phenotypic changes by transmission of protein misfolding have been found in yeast and other fungi. In these organisms, the proteins Sup35, Ure2, HET-S and Rnq1 can adopt alternative conformations with different activities, and the misfolded protein aggregates can convert the monomeric folded proteins *in vitro* and *in vivo*[34,35].

Although all these findings support the idea that the molecular cause of neurodegenerative diseases is the accumulation of misfolded and aggregated protein, the final proof of this hypothesis would be to cure the disease in humans by arresting or reversing the protein conformational and aggregational changes.

Determinants of misfolding and aggregation

There is no evident sequence or structural homology among the proteins that have been implicated in protein conformational disorders. However, from the pioneering electron microscopic studies by Cohen[36] to the recent detailed structural studies by Serpell and Blake[37,38], there is accumulating evidence that the aggregates formed by the different misfolded proteins have the same molecular form (FIG. 1).

With the exception of SOD1 in ALS, the misfolding and aggregation of the proteins implicated in neurodegenerative diseases has been modelled *in vitro*. Low-resolution structural studies have shown, in all cases, a large secondary structural difference between the monomeric native protein and the aggregated material[39–42] (FIG. 2). In general, the native conformation is composed of α-helical and unordered structures, whereas the misfolded protein conformation is rich in β-SHEETS. Because of their insolubility and non-crystalline nature, high-resolution studies of

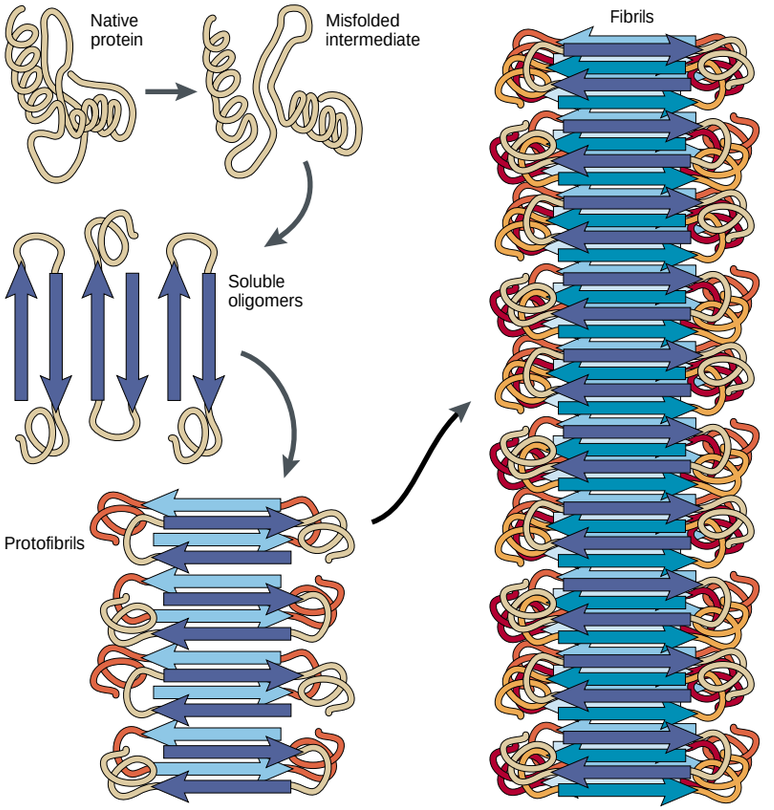

Figure 2 | **Schematic representation of the pathway leading to protein misfolding and aggregation.** The natively folded protein, normally produced in diverse cell types, adopts a random coil or α-helical conformation. In the elderly brain, the first pathological step would be the formation of a misfolded intermediate that exposed to the aqueous environment hydrophobic fragments that are normally buried inside the protein. This intermediate has a high tendency to aggregate and become stabilized, in a rate-limiting process, by the formation of an oligomeric β-sheet structure, which by incorporation of additional monomers gives rise to protofibrils and finally to cross-β amyloid-like fibrils.

aggregated proteins have been difficult. But recent studies using X-ray fibre diffraction and solid-state nuclear magnetic resonance have confirmed the β-sheet-rich structure of protein aggregates implicated in neurodegenerative diseases[38,43]. An exception appears to be the structure of tau aggregates, which is composed mainly of α-helices, as shown by studies using circular dichroism and fourier-transformed infrared spectroscopy[44].

These studies have resulted in a molecular model of amyloid-like fibrils composed of several protofilaments, which consist of hydrogen-bonding β-sheet structures with the β-strands running perpendicular to the long fibre axis, a structure known as a cross-β conformation (FIG. 2). It is clear from these structural studies that a large conformational rearrangement of the polypeptide chain occurs during misfolding and aggregation. However, it is not known whether the misfolding triggers protein aggregation, or protein oligomerization induces the conformational changes[3]. On the basis of the available evidence, it is likely that slight conformational changes result in the formation of a misfolded intermediate, which is unstable in an aqueous environment because of the exposure of hydrophobic segments to the solvent[2,3]. This unstable intermediate is stabilized by intermolecular interactions with other molecules, forming small β-SHEET OLIGOMERS which, with further growth, produce amyloid-like fibrils (FIG. 2). In this model, the conversion of the folded protein into the pathological form is triggered by structural changes, but complete misfolding depends on oligomerization.

Studies using different solution conditions and protein sequence modifications have been useful for understanding the structural requirements of conformational changes that result in aggregation. Amyloid formation by the Alzheimer's Aβ protein is the most extensively studied[45]. Peptides containing the 40- or 42-residue forms of Aβ, and shorter derivatives, form amyloid-like fibrils *in vitro*, which are morphologically, tinctorially, immunologically, spectroscopically and ultrastructurally similar to fibrillar aggregates extracted from AD amyloid plaques[46–49]. Studies using shorter Aβ fragments or mutated peptides have shown that the internal hydrophobic region between amino acids 17 and 21 is the most important for the early steps of Aβ misfolding and aggregation, indicating that Aβ assembly is partially driven by hydrophobic interactions[47,49,50]. This idea is consistent with the higher ability of Aβ peptides to aggregate with two or three extra hydrophobic amino acids at the carboxyl terminus[48]. Similar studies of PrP misfolding have identified the hydrophobic fragment 106–126 as the most relevant for protein aggregation[51]. Although less is known about the process of α-synuclein fibrillogenesis, the evidence indicates that the amino-terminal fragment 1–87 might be crucial[43]. This fragment contains the hydrophobic non-amyloid component peptide that was previously identified as a component of AD amyloid-like plaques by Saitoh and co-workers and was shown to be amyloidogenic[52].

The finding that hydrophobic sequences are critical for aggregation of Aβ, PrP and α-synuclein indicates that protein aggregation is driven by the exposure of hydrophobic residues to the surface of the misfolded protein. However, huntingtin and other polyglutamine-containing proteins seem to differ. In HD, SCA and other polyglutamine diseases, both disease and protein aggregation are associated with an inherited expansion of CAG (the codon for glutamine) repeats[53]. Aggregation of huntingtin *in vitro* depends on the length of the polyglutamine repeat[54]. The glutamine has an amide group that provides a polar side chain and the potential to form a hydrogen bond with water. An alternative model to explain the aggregation of polyglutamine-containing proteins is based on 'polar zipper' interactions between proteins[55]. In this model, β-sheets are formed and stabilized by the collective strength of cooperative hydrogen bonding involving the amide group of the glutamine residue. The aggregation of the yeast prions seems to follow the same principle[56]. Therefore, similar fibrillar products can arise from two different (and, in some respects, opposite) driving forces: hydrophobic interactions or polar hydrogen bonding among side-chain groups.

β-SHEET OLIGOMERS
Structures containing several units of a protein organized in a β-pleated sheet conformation.

Box 1 | Protein aggregation and selective neuronal vulnerability

One of the most intriguing aspects of the pathology of neurodegenerative diseases is the selective vulnerability of neuronal populations to damage in the different diseases. The brain region and the class of neurons affected determine the clinical symptoms that are associated with each disease. Cerebral damage in Alzheimer's disease (AD) patients occurs mainly in the hippocampus, entorhinal cortex and neocortex, resulting in dementia. In Parkinson's disease (PD), the cells of the substantia nigra die, leading to rigidity and tremor. Huntington's disease (HD) causes neurodegeneration in the striatum (caudate nucleus and putamen) and results in uncontrolled movement. Spinocerebellar ataxia affects mainly the cerebellum, leading to ataxia. In amyotrophic lateral sclerosis (ALS), cell death occurs in the spinal cord, brain stem and areas of the motor cortex, causing progressive paralysis. The relationship between the brain areas affected and the clinical symptoms is clearest in the early stages of the diseases, whereas in the later stages there is usually more extensive damage and a wider range of symptoms. For example, AD patients frequently develop symptoms of PD later on. Also, many patients with HD, PD and ALS can develop dementia, reflecting cortical neurodegeneration later in the disease.

The finding that diverse protein aggregates accumulate mainly in the brain areas that show degeneration is a further indication of the key role of misfolding and aggregation in the pathogenesis of neurodegenerative diseases. In the framework of the protein misfolding hypothesis of brain degeneration, the crucial question is why different proteins are more prone to misfold, aggregate and accumulate in distinct brain regions, thereby leading to such specific patterns of neurodegeneration. We can only speculate about the answer. Local changes in expression or in post-translational modifications of the protein that is misfolded might explain the regional selectivity of protein aggregation. Regional changes in the concentration or activity of chaperone proteins, or other factors that specifically control the folding of certain proteins, might also be involved. For extracellular aggregates, differential localization of cellular receptors that can anchor specific types of fibril might explain the selective accumulation of amyloid-like plaques. Another potential mechanism involves selective alterations in clearance pathways that will remove different misfolded and aggregated proteins. Finally, certain neurons might be more sensitive to apoptotic stimuli triggered by different misfolded proteins, leading to specific cellular damage, which in turn results in inefficient removal of protein aggregates from these cells.

The mechanism of misfolding and aggregation

Several genetic and environmental factors have been associated with protein misfolding and aggregation. The mechanism by which mutations lead to conformational changes and disease is probably destabilization of the normal protein conformation, favouring misfolding and aggregation. Environmental factors that might catalyse protein misfolding include changes in metal ions, pathological chaperone proteins, pH or oxidative stress, macromolecular crowding and increases in the concentration of the misfolding protein[3,45]. Many of these alterations are associated with ageing, consistent with the late onset of neurodegenerative diseases[57].

Kinetic studies have shown that the aggregation of Aβ, PrP, huntingtin, α-synuclein and other proteins involved in systemic amyloidosis follows a seeding/nucleation mechanism[48,58,59], which resembles a crystallization process. The critical event is the formation of protein oligomers that act as a nucleus to direct further growth of aggregates. Nucleation-dependent polymerization is characterized by a slow lag phase in which a series of unfavourable interactions forms an oligomeric nucleus, which then rapidly grows to form larger polymers. The lag phase can be minimized or removed by addition of pre-formed nuclei or seeds.

APOPTOSIS
The process of programmed cell death, characterized by distinctive morphological changes in the nucleus and cytoplasm, chromatin cleavage at regularly spaced sites, and the endonucleolytic cleavage of genomic DNA.

At least two intermediates have been identified in the pathway from the native monomeric protein to the fibrillar fully aggregated structure *in vitro* (FIG. 2), mostly in studies of Aβ[45]. The first are soluble, low-molecular-weight oligomers (dimers to decamers), which have been found in test-tube experiments, in the conditioned medium of cells that constitutively secrete Aβ, in human cerebrospinal fluid and in human brain homogenate[60–63]. Structural and biochemical characterization of these intermediates has been challenging because they are transient and unstable. The second intermediates are short, flexible, rod-like structures termed protofibrils, which have been studied by electron microscopy, photon correlation spectroscopy and atomic force microscopy[64,65]. Protofibrils are unbranched polymers that are 3–6 nm wide and up to 100 nm long. Kinetic studies have shown that they are metastable intermediates that elongate by coalescence of smaller protofibrils with a rate that depends on Aβ concentration, temperature, ionic strength and pH[66]. Protofibrils are in a dynamic equilibrium with oligomeric Aβ and are the direct precursor of amyloid-like fibrils[65]. Secondary structure studies show that protofibrils have a high β-sheet content, like fibrils, and can bind the amyloid-specific dyes Congo red and thioflavin T[65]. Protofibrils have also been detected in the aggregation process of α-synuclein[67]. The evidence indicates that these intermediates, the monomeric protein and the fibrillar aggregates, are present simultaneously and in a dynamic equilibrium[45,60].

Mechanisms of neuronal death

Selective neuronal loss, synaptic alterations and neuroinflammation (reactive astrocytosis and activated microglia) are typical features of neurodegenerative diseases[4]. However, the region of the brain that is most affected differs among diseases and determines the clinical symptoms of each (BOX 1). Neuronal death in AD occurs mainly in regions of the brain that are implicated in memory and abstract thinking, such as the hippocampus, amygdala, entorhinal cortex and association areas of the neocortex[68]. PD is characterized by neuronal loss in the substantia nigra and depletion of dopamine in the striatum[7]. In HD, there is severe neuronal loss initially in the neostriatum and later in the cerebral cortex[69]. The cell death process in ALS is relatively selective for lower motor neurons in the spinal cord and brainstem, and for upper motor neurons in the motor cortex[70]. In TSEs, the location and extent of neuronal loss vary[71], and spongiform degeneration of brain tissue is the salient feature of the pathogenesis.

Neuronal loss in neurodegenerative diseases occurs by programmed cell death or apoptosis[72]. At least three hypotheses have been proposed to explain how protein misfolding and aggregation might be associated with neuronal APOPTOSIS (FIG. 3).

The loss-of-function hypothesis. In this view, neurodegeneration is caused by the loss of normal activity of the protein, which is depleted during misfolding and aggregation. This model has been mainly considered for

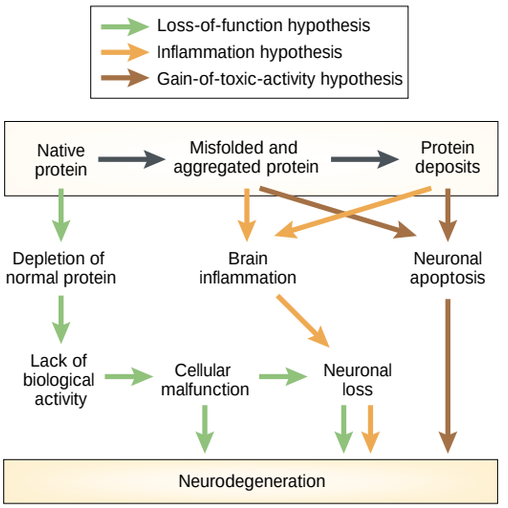

Figure 3 | **Models for the mechanism of neurodegeneration associated with protein misfolding and aggregation.** Three models have been proposed. Although the beginning and the end of the process are the same in the three hypotheses, the events that induce neuronal death are different. In the loss-of-function model, the lack of activity of the native protein is the key step, whereas in the gain-of-toxic-activity hypothesis, the crucial process is the neurotoxicity of the misfolded and/or aggregated protein. In the inflammation model, neuronal death is indirectly mediated by activation of astroglial cells. In some diseases, a combination of these mechanisms might operate.

TSE, HD and ALS. In ALS, the fact that the protein that is misfolded (SOD1) catalyses the conversion of toxic superoxide anions to hydrogen peroxide was initially used to support the loss-of-function hypothesis, as depletion of SOD1 by misfolding and aggregation could lead to accumulation of superoxide radicals. However, SOD1 knockout mice show no degeneration of motor neurons[73], and variations in enzymatic activity and half-life of different SOD1 mutants do not correlate with the age of onset or the severity of the disease[74]. Overexpression of some SOD1 mutations in transgenic mice causes motor neuron disease despite unaltered or elevated levels of SOD1 activity[24].

In HD, the fact that homozygous knockout mice for huntingtin die early in embryonic development has been used as evidence for the loss-of-function hypothesis[75]. However, heterozygous mice reach adulthood with a normal phenotype. A conditional knockout animal, in which huntingtin expression was arrested in the brains of adult mice, showed motor deficits, hypoactivity, slight tremor and brain degeneration in the striatum and cortex[76]. Some of these alterations are similar to those found in HD patients, indicating that a loss of huntingtin function might be implicated in HD pathogenesis. Several biological activities have been attributed to wild-type huntingtin, including cytoskeletal anchoring, axonal transport, endocytosis and intracellular trafficking[75]. Evidence also points to a role in brain development and neuroprotection. Huntingtin is a CASPASE substrate that is actively cleaved during apoptosis in cultured cells, and *in vitro* experiments have shown that it can protect neurons from a variety of apoptotic stimuli, including serum withdrawal, death receptors and pro-apoptotic proteins[75]. The anti-apoptotic activity of huntingtin is downstream of the pro-apoptotic Bax (Bcl2-associated X protein) protein family and upstream from the caspase-3 apoptotic executor. However, a strong argument against the loss-of-function hypothesis is that patients who are homozygous and heterozygous for huntingtin mutations have similar clinical features.

PrP^C has been indicated to be involved in signal transduction, as it is found in caveolae-like domains and interacts with several intracellular signalling proteins[77]. *In vitro*, ligand-activation of PrP^C leads to a neuroprotective signal[78]. In addition, PrP-null neuronal cell lines are more susceptible to serum deprivation-induced apoptosis, and overexpression of the anti-apoptotic protein Bcl2 (B-cell leukaemia/lymphoma 2) can attenuate this sensitivity[79]. PrP fusion proteins interact with Bcl2 (REF. 80), and the highly conserved octapeptide repeats of PrP are similar to the Bcl2 homology domain 2 (BH2) of Bcl2 family proteins. This domain is crucial for the anti-apoptotic function of Bcl2 and its interaction with the pro-apoptotic protein Bax. Moreover, PrP^C can protect human neurons against Bax-induced apoptosis[81]. These results indicate that loss of PrP^C function might contribute to TSEs by altering the signalling pathways related to neuronal survival.

Similar anti-apoptotic activities have been reported for APP and α-synuclein *in vitro*[82,83], indicating that an imbalance between pro- and anti-apoptotic activities might be associated with neurodegenerative diseases. However, APP and α-synuclein knockout mice show no signs of neurodegeneration[84,85]. PrP-null mice are viable[86], but contradictory results have been reported on their phenotype[87]. Some studies found no significant abnormalities whereas others reported impairments in neuronal functioning, loss of cerebellar Purkinje cells and defects in sleep patterns and circadian activity[87]. The divergences among PrP-null mice arise from differences in the methods used to suppress protein expression, and more specifically whether the expression of an adjacent PrP homologue protein, Doppel, has been artificially induced in the brain[87]. A recent study showed that post-natal PrP knockout mice remained healthy, without evidence of neurodegeneration or other histopathological changes, for up to 15 months after the knockout, diminishing the likelihood that loss of PrP function cause TSEs[88].

Several of the proteins implicated in misfolding (including PrP, SOD1 and Aβ) can bind metal ions, indicating that a common mechanism of neurodegeneration might involve changes in metal ion homeostasis[89]. PrP, SOD1 and Aβ have been reported to have a superoxide dismutase activity *in vitro*[89], indicating that an abnormal reaction between a protein and a redox-active metal ion (copper or iron) might promote the formation of reactive oxygen species or radicalization, and lead to neurodegeneration.

The gain-of-toxic-activity hypothesis. The most widely accepted theory of brain degeneration in neurodegenerative diseases proposes that misfolding and aggregation results in the acquisition of a neurotoxic function by the misfolded protein. This concept is based on direct induction of neuronal apoptosis by aggregates of several misfolded proteins (or fragments of the proteins) *in vitro*[90–93]. Additional support for this hypothesis comes from experiments with transgenic animals in which incorporation of the human mutated gene encoding the misfolded protein can trigger neurodegeneration. There is also evidence that β-sheet oligomerization of non-disease-related proteins is cytotoxic, indicating that misfolding and aggregation of any protein results in inherent toxicity[94]. This concept might provide a unifying mechanism of cell death and tissue degeneration in protein misfolding disorders.

Several mechanisms have been proposed for the neurotoxic activity of misfolded aggregates (FIG. 4), and it is likely that different pathways operate depending on whether the proteins accumulate intra- or extracellularly. Extracellular aggregates might activate a signal transduction pathway that leads to apoptosis by interacting with specific cellular receptors. For example, RAGE (receptor for advanced glycation end products), a multi-ligand immunoglobulin superfamily cell-surface molecule, binds amyloid-like fibrils comprising Aβ, PrP, amylin and amyloid-A, thereby inducing cellular stress and activation of NF-κB[95]. Intracellular aggregates might damage cells by recruiting factors that are essential for cell viability into the fibrillar aggregates. Components of the proteasome, chaperone proteins, cytoskeletal proteins and transcription factors have been found in huntingtin and α-synuclein aggregates[96,97]. Another proposed mechanism for Aβ and PrP neurotoxicity is membrane disruption and depolarization mediated by ion-channel formation, resulting in alteration of ion homeostasis and disregulation of cellular signal transduction, leading to cell death[98,99]. Finally, protein aggregates might induce OXIDATIVE STRESS by producing free radical species, resulting in protein and lipid oxidation, elevation of intracellular calcium and mitochondrial disfunction[100,101].

The hypothesis that aggregates are toxic has been challenged by recent histopathological, biochemical and cell biology studies. Neuropathological analysis of the brains of people affected by PD or AD have shown that neurons containing Lewy bodies or neurofibrillary tangles seem to be healthier, by morphological and biochemical analysis, than neighbouring cells[102,103]. In addition, amyloid-like plaques and Lewy bodies are found in people without evident neuronal loss or clinical signs of AD or PD[7,16]. Moreover, in some animal models of AD, TSE, HD and ataxias, cerebral damage and clinical symptoms have been detected before protein aggregates[28,29]. Mice in which the endogenous ataxin-1 protein contains extra glutamine repeats show many clinical and pathological signs similar to SCA type 1, including loss of motor coordination and Purkinje cell degeneration[104]. However, mutant ataxin-1 was most soluble in the brain regions that were most affected, indicating that neurons that cannot capture the mutant protein in aggregates suffer the worst damage[104]. Finally, although in most studies inhibitors of protein aggregation also prevent neuronal damage, some *in vitro* and *in vivo* studies found that, under certain conditions, prevention of aggregation did not inhibit (or even increased) cell death[29,62,105]. These findings indicate either that there is not a unifying mechanism of neurodegeration in these diseases or that the process of misfolding and early stages of oligomerization (FIGS 2 and 3), rather than deposition of the mature compacted aggregates in the brain, are the real culprits in neurodegeneration[106,107].

The latter hypothesis is supported by results showing that purified oligomeric species and protofibrils have similar or even greater toxicity than mature amyloid-like fibrils[62,63,94,106]. Some investigators have proposed that the formation of amyloid-like fibrils might be a protective mechanism to sequester and isolate toxic misfolded intermediates[104,105,107]. Alternatively, both soluble misfolded intermediates and amyloid-like fibril deposits might be toxic, but perhaps by different mechanisms. For example, soluble oligomeric species might induce a signalling pathway that leads to apoptosis, whereas amyloid-like plaques might take up tissue space, break down neuronal connections and recruit essential cellular factors. In addition, the concept that protein deposits are static and irreversible structures has been changing to accommodate recent results showing that the protein component of aggregates, as well as the associated proteins, are in dynamic

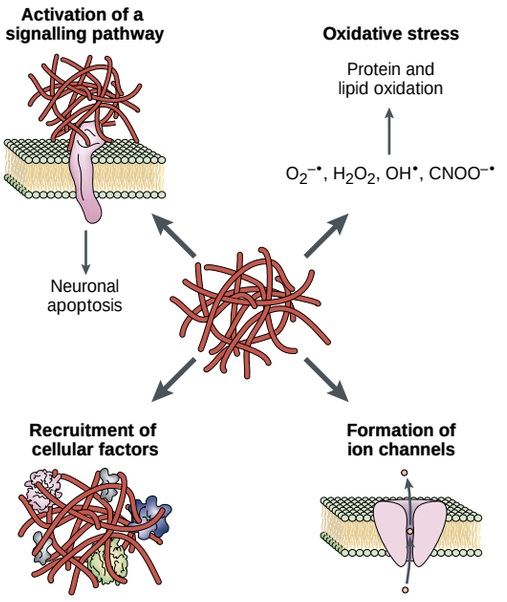

Figure 4 | **Models for the neurotoxic mechanism of misfolded aggregates.** At least four hypotheses have been proposed to explain the mechanism of neurotoxicity associated with protein misfolding and aggregation: activation of an apoptotic signalling pathway, recruitment of essential cellular factors, formation of ion channels and the induction of oxidative stress.

CASPASES
A family of intracellular cysteine endopeptidases that have a key role in inflammation and mammalian apoptosis. They cleave proteins at specific aspartate residues.

OXIDATIVE STRESS
A disturbance in the pro-oxidant–antioxidant balance in favour of the former, leading to potential cellular damage. Indicators of oxidative stress include damaged DNA bases, protein oxidation and lipid peroxidation products.

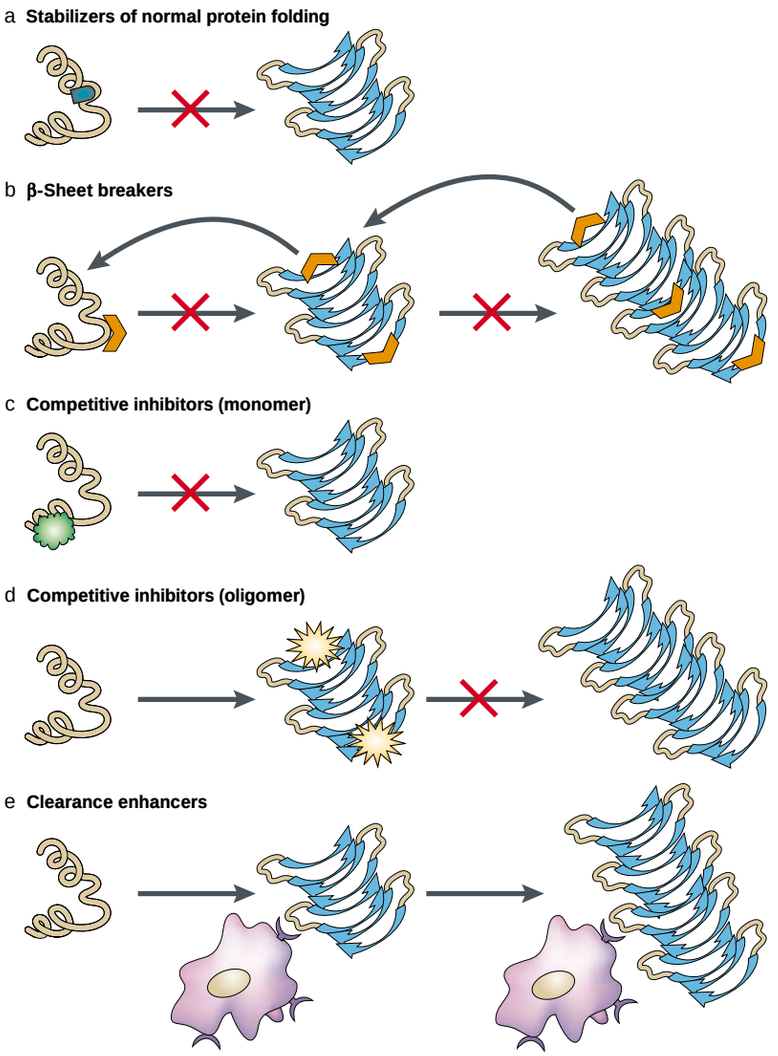

Figure 5 | **Schematic representation of different therapeutic strategies to arrest protein misfolding and aggregation. a** | Stabilization of the folding of the native protein. **b** | Inhibition and reversal of protein misfolding by compounds that can specifically destabilize β-sheet structures. **c** | Competitive inhibition of protein oligomerization by compounds that bind to the monomeric protein. **d** | Competitive inhibition of aggregation by molecules that bind to aggregated β-sheets and block further incorporation of monomers. **e** | Increased clearance of the misfolded/aggregated protein by compounds that boost clearance mechanisms or decrease the stability of protein aggregates.

equilibrium with the soluble versions of the proteins[45,108,109], making it even more difficult to understand the contributions of diverse mechanisms to neurodegeneration.

The brain inflammation hypothesis. In this hypothesis, abnormal protein aggregates act as irritants and cause a chronic inflammatory reaction in the brain that leads to neuronal death and synaptic changes[110,111]. Evidence that there is a chronic inflammatory reaction in the brain for most of the neurodegenerative diseases includes: extensive astrocytosis and microglial activation in patients, especially around protein deposits[112–116]; specific accumulation of inflammatory proteins in cerebral protein aggregates, including complement proteins, complement inhibitors, acute-phase reactants, inflammatory cytokines, proteases and protease inhibitors[110]; increased levels of inflammatory proteins such as cytokines, chemokines and growth factors in the brain[110,117]; *in vitro* studies showing that misfolded and aggregated proteins can activate microglia and astrocytes, inducing them to release inflammatory proteins[118,119]; and retrospective analysis of clinical trials showing a decrease in the incidence of AD by treatment with nonsteroidal anti-inflammatory drugs in animal models and humans[120].

Other studies have led to the idea that inflammation might be beneficial in these diseases[111]. Inhibiting the activation of the C3 complement component increased neurodegeneration and plaque load in transgenic animal models of AD[121]. In addition, boosting the immune system by vaccination with Aβ resulted in a marked reduction in cerebral amyloid plaques[122] and improvement of behavioural and cognitive impairments in animal models[123,124]. However, when this approach was used in humans, several cases of MENINGOENCEPHALITIS were observed, some of them having elevated white blood cell counts in the cerebrospinal fluid, indicating a central inflammatory reaction[125]. These findings reinforce the idea that brain inflammation might be a double-edged sword in neurodegenerative diseases, having both negative and positive effects simultaneously[111].

A common therapy for conformational diseases?

Despite impressive progress in understanding the pathogenesis of neurodegenerative diseases, none of these disorders can be successfully treated. If protein misfolding and aggregation are central events in the pathogenesis of neurodegenerative diseases, a therapy directed to the cause of the illness should aim to correct protein misfolding. At least four approaches have been proposed to attack protein misfolding and aggregation (FIG. 5): stabilization of the native protein conformation; inhibition and reversion of protein conformational changes; competitive inhibition of protein oligomerization; and increased clearance of the misfolded protein.

Stabilization of the native folding of the normal protein isoform (FIG. 5a) in prion-infected neuroblastoma cells by treatment with chemical chaperones (reagents known to stabilize native conformation of proteins) prevents PrP misfolding[126]. Small molecules that bind to the native protein and stabilize its structure have been reported for Aβ[127] and transthyretin (a protein that has been implicated in systemic amyloid disorders)[128].

Protein engineering has been proposed as an approach to create sequence-modified proteins with higher stability, lower tendency to misfold and the ability to trans-suppress the aggregation of wild-type proteins[129]. Although the idea is attractive, its therapeutic application would require sophisticated gene therapy technologies. A natural example of this has been observed in transthyretin-related amyloidosis, in which a spontaneous stabilizing mutation (T119M) has been described in some families. Heterozygote patients that have both the stabilizing T119M and pathogenic (V30M)

MENINGOENCEPHALITIS
An inflammatory process involving the brain and meninges, most often produced by pathogenic organisms that invade the central nervous system, and occasionally by toxins, autoimmune disorders and other conditions.

mutations show slower disease progression[130]. *In vitro* studies of the T119M mutant protein showed that the substitution stabilizes the native structure of transthyretin, decreasing its amyloidogenesis[131]. The protein containing the stabilizing mutation can also prevent the misfolding and aggregation of wild-type and pathogenic mutants of transthyretin[132]. The same concept has been applied to prevent prion replication in transgenic mice[133]. Animals expressing mouse PrP bearing two modifications corresponding to sheep polymorphisms that have been shown to protect against scrapie, showed a dominant-negative inhibition of prion propagation. However, the exact mechanism of protection is not known and alternative explanations to the over-stabilization concept cannot be ruled out. Together, these findings indicate that over-stabilized variants of proteins might prevent protein misfolding and aggregation.

The over-stabilization concept might be combined with simultaneous destabilization of the pathological β-sheet conformation of misfolded proteins, for example by using β-sheet breaker peptides to inhibit and reverse protein misfolding (FIG. 5b). β-Sheet breakers are short synthetic peptides containing the self-recognition motif of the protein undergoing misfolding and are engineered to arrest the folding of the polypeptide chain in a β-sheet structure[3]. β-Sheet breaker peptides have been designed to block the conformational changes and aggregation of both Aβ and PrP[134–136]. They can inhibit and reverse protein misfolding and aggregation, and might be able to be used both prophylactically and therapeutically. IDOX (4′-iodo-4′-deoxydoxorubicin) and tetracycline have also been reported to inhibit protein misfolding and to dissolve pre-formed aggregates of several proteins[137,138], showing that the reversal of protein misfolding and aggregation is a feasible target.

Compounds that can competitively block protein–protein interactions are most often used to inhibit aggregation. Two classes of competitive inhibitor can be designed: compounds that block the interaction between monomers (FIG. 5c) and molecules that stack at the edges of β-sheet aggregates, preventing their growth (FIG. 5d). Experimentally, it is difficult to determine the mechanism of action of competitive inhibitors. This is an important problem, because depending on which is the most toxic species in the aggregation pathway, it is likely that some inhibitors might lead to accumulation of toxic intermediates and thus might aggravate the disease. Several small molecules with an affinity for intermolecular β-sheets (such as Congo red), fragments of the proteins undergoing misfolding, specific antibodies and proteins capable of interacting with β-sheet aggregated proteins (such as apolipoprotein E, amyloid P-component and proteoglycans) have been reported to prevent protein aggregation[139–141].

Increasing the clearance of misfolded and aggregated proteins (FIG. 5e) is an interesting approach that has been based on findings showing that accumulation of protein aggregates depends on a balance between deposition and clearance. In a conditional transgenic model of HD, preventing the production of mutant fragments caused nuclear inclusions to disappear, indicating that cells can metabolize the aggregated material[142]. Perhaps the most promising strategy to increase the clearance of misfolded proteins is the immunization approach, which was first described for AD[122]. Aggregates of synthetic Aβ protein were used as antigens to induce the immune system to produce antibodies to clear them. Immunization can reduce amyloid load, cerebral damage and behavioural impairments in transgenic animal models of AD[122–124]. A similar approach has been used for the treatment of TSE[143]. However, a clinical trial to evaluate the efficacy of the immunization strategy in humans affected by AD was stopped owing to several cases of meningoencephalitis[125]. Little is known about the reasons for this side effect, but future research should bring more knowledge about this problem and might result in new strategies to minimize brain inflammation after vaccination. Immunization with fragments of Aβ that preferentially stimulate the B cells (and not the T cells), or passive immunization with antibodies against the misfolded proteins, might be safer strategies[125].

Enhancement of the clearance of amyloid-like deposits has been also attempted by removing some accessory constituents found in plaques. The underlying idea is that other factors that bind tightly to the aggregates might increase their insolubility and resistance to proteolytic degradation. Strategies for the specific removal of amyloid-P components, proteoglycans and metal ions have shown the best results in models of AD and systemic amyloidosis[144–146].

Conclusions and perspectives

Research over the past 5 to 10 years has provided evidence for a common mechanism of neurodegeneration in which the critical events are the misfolding, aggregation and accumulation in the brain of otherwise normal proteins. Each neurodegenerative disease is associated with abnormalities in the folding of a different protein, but the molecular pathways leading to misfolding and aggregation, and the mechanisms by which this process might lead to neuronal death, seem similar. These findings provide hope for a common therapeutic strategy to treat these illnesses. The development of a therapy based on the misfolding concept would also demonstrate the role of protein conformational changes as a triggering event in the pathology and help to elucidate which of the different putative pathways relating protein misfolding and aggregation with neurodegeneration is correct.

Perhaps the most exciting direction for future research is to investigate whether other diseases are associated with similar alterations in protein folding and aggregation, and whether this process is only linked to disease or is a more general phenomenon. For many years it was thought that misfolding and formation of amyloid-like aggregates was specific to particular, unusual proteins. However, recent studies have shown that many (if not all) proteins can form amyloid-like structures under appropriate conditions[147,148]. In addition, the finding that the mammalian prion protein and several yeast proteins[34,35] can propagate biological information through transmission of protein conformational

changes from one molecule to another, has opened a new area in biology. Transmission of biological information through alternative protein folding might represent a natural and rapid way to modify the structure and activity of proteins. This modification might allow organisms to adapt to challenging environments and thus evolve within a single generation without the need for genetic changes. The possibility that many proteins can adopt multiple conformations to exert different functions might revolutionize our understanding of biology.

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α-synuclein | amylin | apolipoprotein A-II | apolipoprotein E | APP | ataxin-1 | Bax | Bcl2 | Doppel | HET-S | huntingtin | NF-κB | prion protein | RAGE | Rnq1 | SOD1 | Sup35 | transthyretin | ubiquitin | Ure2

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